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Establishment of Microbial Population in Sterile Mine Spoils and Overburden.

II Amendments Required for Acid Mineral-Mine Spoils

ESTABLISHMENT OF MICROBIAL POPULATIONS IN STERILE MINE SPOILS AND OVERBURDEN

II. AMENDMENTS REQUIRED FOR ACID MINERAL-MINE SPOILS

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS ii

LIST OF TABLES iv

LIST OF FIGURES v

ABSTRACT vii

INTRODUCTION 1

MATERIALS AND METHODS 3

Subsoil and Spoil Sample Preparation 3

Soil Chemical Test and Bioassay Response Parameters . 3

Experimental Design, Bioassay 3 7

RESULTS 11

Overview of Results 11

Microbial Responses 14

Growth Parameter Interrelationships 21

THE FATE OF PHOSPHORUS IN THE SPOIL AND ITS EFFECT ON GROWTH RESPONSE KINETICS 25

Phosphorus Availability 25

Phosphorus Growth Kinetics 30

DISCUSSION 37

Microbial Bioassays 37

Microbial Role in Fertilization 39

SUMMARY AND CONCLUSIONS 42

LITERATURE CITED 44

APPENDIX A 46

LIST OF TABLES

Table Page

1 Fractionation of "typical" surficial spoil material . 4

2 Experimental design of bioassay 3 8

3 Initial conditions for Bioassay 12

4 Values of μ (days^{-1}) for the bioassay at 10.5 days and at 30 days 34

A-1 T-test statistical comparison of total nitrogen content of the various treatments in bioassay 3 . . 46

A-2 T-test statistical comparison of nitrogen fixation rates of the various treatments in bioassay 3 . . 47

A-3 T-test statistical comparison of chlorophyll *a* content of the various treatments in bioassay 3 47

A-4 T-test statistical comparison of dehydrogenase activity of the various treatments in bioassay 3 . . 48

LIST OF FIGURES

Figure Page

1 Graphical representation of spoil fractionation . . 5

2 Growth responses at three weeks and six weeks for each treatment 13

3 Chlorophyll *a* as a function of bicarbonate extractable phosphorus 15

4 Dehydrogenase activity as a function of bicarbonate extractable phosphorus with best fit power curve . . 18

5 Nitrogen fixation as a function of the bicarbonate extractable phosphorus content with best fit power curve 20

6 Relationship of chlorophyll *a* to nitrogen fixation . 22

7 Relationship of dehydrogenase activity to nitrogen fixation 23

8 Relationship of dehydrogenase activity to chlorophyll *a* content 24

9 Equilibrium diagram relation pH to the relative concentration of the four phosphate species at C_T = 10^{-3} moles/liter (*Jurinak*, 1976, personal communication, Utah State Univ.) 26

10 Diagram showing the classification of phosphate compounds in three major groups. Fertilizer phosphates are generally in the "readily available phosphate" group but are quickly converted to the slowly available forms. These can be less available and are then classed as very slowly available. At any one time perhaps 80-90 percent of the soil phosphorus is in "very slowly available" form since perhaps less than one percent would be expected to be readily available (*Buckman and Brady*, 1969) 26

11 The relationship of HCO_3^- extractable phosphorus to phosphorus addition (as Na_2HPO_4) at various liming levels. Equilibrium time before extraction equaled three days 28

12 A graphical representation of the availability of phosphorus in the bioassays for various treatments . 29

LIST OF FIGURES (CONTINUED)

Figure Page

13 Model showing various compounds controlling the solubility of $H_2PO_4^-$ (moles/liter) as a function of pH (*Jurinak*, 1976, personal communication, Utah State University, Logan) 31

14 A plot of estimated growth rate versus the extractable phosphorus content at 10.5 days 36

ABSTRACT

The revegetation of sterile acid mine spoils is a costly and lengthy process due to the many factors (pH, toxic materials, low nutrients, poor soil structure, temperature, moisture, length of growing season, etc.) that inhibit the establishment of stable plant communities. A microbial bioassay technique applied to soil systems has been developed to allow a rapid (four weeks) and integrated (with respect to nutrients and toxicants) determination of the best amendments for stabilizing mine spoils by revegetation. Using mine spoil from the Blackbird Mine, Cobalt (near Salmon), Idaho, various combinations of nitrogen, phosphorus, chelators, trace elements, potassium, manure and salt leaching were studied with the bioassay by microscopal observation and by measurement of nitrogen fixation and accumulation, dehydrogenase activity, chlorophyll *a* accumulation, and other chemical parameters.

It was concluded that the limiting factors for microbial (algal) growth were (in order of importance): soil moisture, pH control, and phosphorus. Other treatments failed to show statistically significant better results over the control. The fate of phosphorus in the spoil and its effect on growth response kinetics is also speculatively discussed.

INTRODUCTION

Mine spoil revegetation processes are believed to be enhanced and/or signified by the presence of microbial indicators; that is, revegetation of soils can take place or has taken place when microbial communities show significant involvement. Acid mine spoils are difficult to revegetate unless multiple changes in the spoil take place that buffer acid produced by bacteria, alter soil texture and insure adequate nutrients and their proper balance (*Chadwick*, 1969; *EPA*, 1971; *Farmer, et al.*, 1976; *Peterson and Nielson*, 1969; *Sorensen, et al.*, 1978). These changes (or amendments) also influence microbial communities as well as the higher plants (grasses, shrubs, and trees) that form the basis of a stable climax community for a given ecosystem.

The microbial community is a complex group of organisms of varying function and is composed of heterotrophic (bacteria, actinomycetes, fungi, protozoa) and autotrophic (soil algae) components. The heterotrophs and chemoautotrophs occur throughout the soil column while the photoautotrophs occur only at the soil surface. Because of their role in binding the soil surface, fixing nitrogen, and producing organic matter, soil algae contribute significantly to soil fertility and are important colonizers of disturbed soil systems. Also their inorganic nutrient requirements are at least qualitatively similar to higher plant systems. Because of their rapid growth rate, a shortterm microbial bioassay could be used to indicate nutrient requirements, screen amendments for their utility in revegetation and define the sequence and timing of limiting factors in mine spoils.

In a previous paper the methodology was developed for assaying microbiological response to amendments applied to a sterile acid (pyritic) overburden mine spoil (Blackbird Mine) in Idaho (*Sorensen, et al.*, 1978). Although the microbial bioassay methodology appeared to have real value in the design of revegetation processes, the following questions remained to be answered:

1. What is the shortest period over which a bioassay provides the necessary results?
2. What are the interrelationships between various measures of microbial response?
3. What is the quantitative impact of potential non-symbiotic nitrogen fixation on the nitrogen fertilizer requirements?
4. Relative to the Blackbird Mine,
    - a. What is the limiting nutrient?
    - b. What special nutrient/mineral relationships affect microbial growth at the Blackbird Mine?
    - c. Do organic forms of nutrients possess special factors that improve microbiological growth?

The objectives of the continued research on the microbial bioassay were to confirm previous results and answer the above questions.

MATERIALS AND METHODS

Subsoil and Spoil Sample Preparation

Spoil material for the bioassay was collected from the copper-cobalt Blackbird Mine in mid-eastern Idaho near Salmon, Idaho. One large sample was gathered from surficial material (1 to 2 ft mean depth) of a "typical" site on the spoils. Subsoil was gathered similarly from the original forest subsoil. Sieve analysis of spoil and subsoil (Table 1 and Figure 1) indicated an adequate fraction of the spoil is soil size material (<2 mm diameter). X-ray diffraction analysis (P. Kolesar, Department of Geology, Utah State University) showed that about 5% of the soils size fraction was illite. The sample can be presumed to contain a significant quantity of ferric and aluminum hydrous oxides as a result of intensive chemical weathering, thus endowing it with clay texture properties. The clay size particles are the major sites of physical-chemical adsorption phenomena as well as microbial activity; both phenomena affect overall nutrient availability.

The fraction that passed a 12 mesh/in sieve (approximate particle size < 1.40 mm) was used in the bioassay. Other methodology and bioassay conditions are described in *Sorensen, et al.* (1978).

Soil Chemical Test and Bioassay Response Parameters

The bioassay was designed based on the results of the first two bioassays described in *Sorensen, et al.* (1978). The methodology for the response parameters is described in detail in *Sorensen and*

Table 1. Fractionation of "typical" surficial spoil material.

% Contained by Weight	Particle Size (P.S.)	Approximate Particle Size Range (mm)
46.9	> 2.5 mesh/in.[a]	> 8.00
13.9	5 mesh/in. < P.S. < 2.5 mesh/in.	8.00 to 4.00
13.7	10 mesh/in. < P.S. < 5 mesh/in.	4.00 to 1.70
1.8	12 mesh/in. < P.S. < 10 mesh/in.	1.70 to 1.40
5.0	24 mesh/in. < P.S. < 12 mesh/in.	1.40 to 0.71
1.0	60 mesh/in. < P.S. < 24 mesh/in.	0.71 to 0.25
0.3	100 mesh/in. < P.S. < 60 mesh/in.	0.25 to 0.15
7.1	250 mesh/in. < P.S. < 100 mesh/in.	0.15 to 0.063
4.3	325 mesh/in. < P.S. < 250 mesh/in.	0.063 to 0.045
2.9	400 mesh/in. < P.S. < 325 mesh/in.	0.045 to 0.038
2.7	P.S. < 400 mesh/in.	< 0.038
99.6	Total	

[a] Tyler Standard Screen scale.

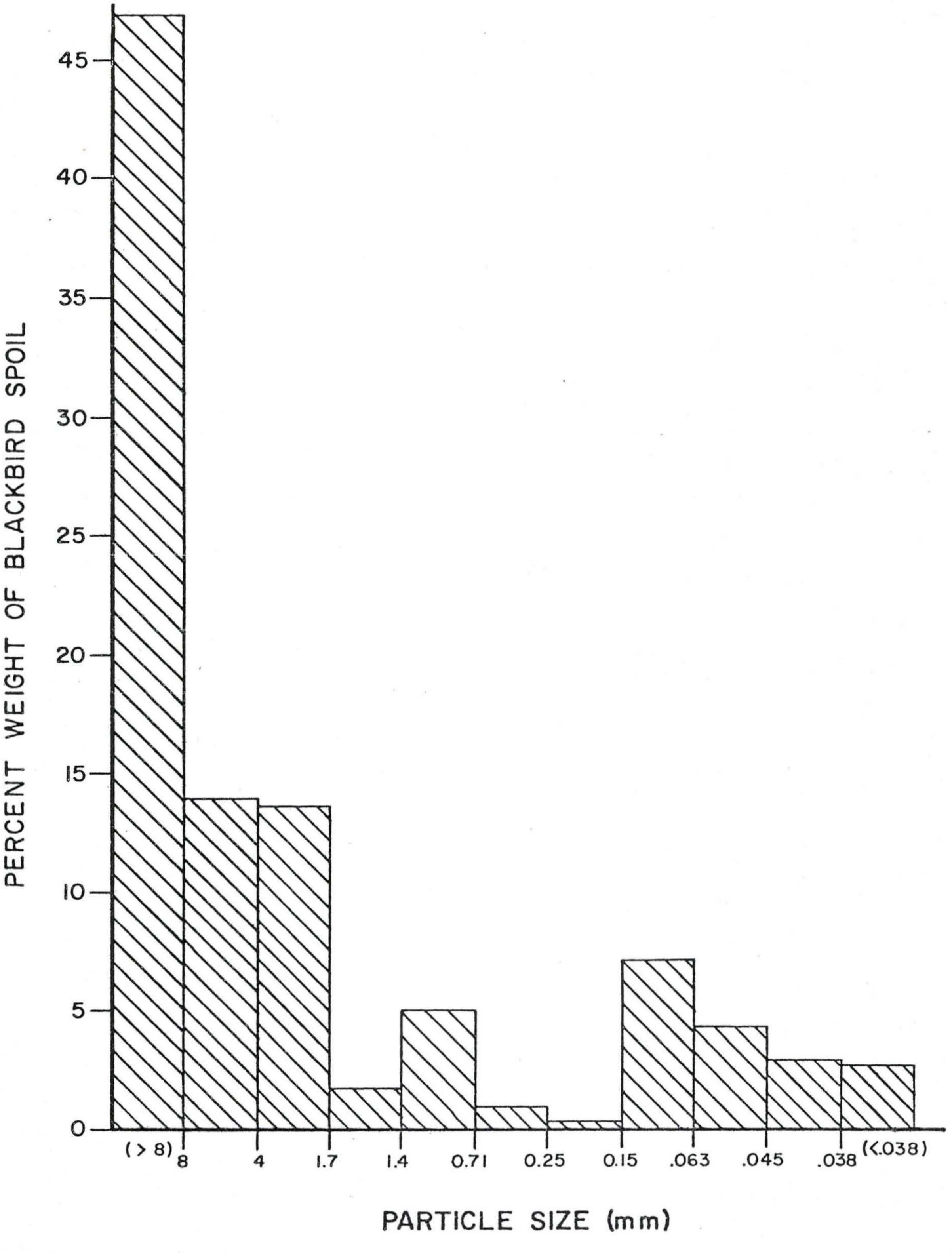


Figure 1. Graphical representation of spoil fractionation.

Porcella (1978) and include:

1. Visual observation with weekly color slides, a pictorial record of macroscopic algal growth.
2. Chlorophyll *a* measurements as a direct chemical evaluation of algal growth.
3. Nitrogen fixation, a blue-green algal response to the nutrients present using the acetylene reduction technique for nitrogenase enzyme activity.
4. The total nitrogen content as an indication of nitrogen accretion from nitrogenase activity (Coleman® Model 29 Nitrogen Analyzer).
5. Dehydrogenase enzyme activity, a measure of microbial respiration.
6. pH, to relate to oxidation of sulfides to produce acids.
7. Bicarbonate extractable phosphorus (plant available) to assess the phosphorus fixing power of the limed spoil.

Initial measurements of the spoil and subsoil included percent saturation, cation exchange capacity, EC_e, total phosphorus, and water soluble metals and anions (Cu, Fe, Mn, Zn, B, Mg, Cu, Na, K, Pb, Co, $SO_4^=$, Cl^-, NO_3^-) with and without lime added.

Organic carbon analysis was attempted using the method of *Walkley and Black* (1934) (see also *Walkley*, 1935). However, this method utilizes a chromic acid oxidation of organic matter which also oxidizes the sulfides of the spoil matrix. Thus, the chemical oxidation methods for soil samples cannot be used for most mine spoil samples. The development of new methods or use of sophisticated instrumentation was precluded by constraints of time and project budget.

Experimental Design, Bioassay 3

Bioassays 1 and 2 tested amendments to both spoil and subsoil material. Equivalent results were obtained with the limed spoil by itself and further testing of subsoil was considered extraneous to the problem at hand, *i.e.*, the nutritional status of the spoil. Therefore subsoil was not studied in Bioassay 3 (Table 2).

CONTROL - Hydrated lime ($Ca(OH)_2$) (*Tisdale and Nelson*, 1975) was added at a level of 0.7 percent (dry weight) to the spoil which gave adequate neutralization for the length of the bioassay.

NITROGEN - Nitrogen was added in Bioassay 1 with only negligible growth resulting. This is about 6 to 7 times the amount added by the USFS (*Farmer, et al.*, 1976).

PHOSPHORUS - Phosphorus was added at various levels in the first bioassays. In both cases it was added in a dry form as either high analysis treble super phosphate ($Ca_3(PO_4)_2$) or $Na_2HPO_4 \cdot 7H_2O$. In Bioassay 3 phosphorus was added as $Na_2HPO_4 \cdot 7H_2O$ to confirm results of the previous bioassays but was added in solution to insure complete mixing for better accuracy. The objective was to determine if phosphorus at 50 and 100 ppm produced increased growth and nitrogen fixation rates over the 25 ppm addition.

POTASSIUM - Potassium was not studied in the first assays. Chemical tests showed the soluble potassium of the spoil to be adequate but it was added here to confirm this hypothesis.

MANURE - Manure was not added in the previous bioassays and sawdust was used in Bioassay 1. There was a low response to the sawdust possibly due to a C:N imbalance and a concurrent lowering of the pH. Fungi did appear to grow well with the sawdust addition. In

Table 2. Experimental design of bioassay 3.

Treatment Number	Treatment[b]	Level of Treatment	Chemical Form	Method of Addition
1	Control (lime)	0.7%	95% $Ca(OH)_2$	Dry addition
2	Nitrogen (N)	0.06%	NH_4NO_3	Added in solution
3	Phosphorus (P)	25 ppm	Na_2HPO_4	Added in solution
4	Phosphorus (P)	50 ppm	Na_2HPO_4	Added in solution
5	Phosphorus (P)	100 ppm	Na_2HPO_4	Added in solution
6	Potassium (K)	0.5%	K_2SO_4	Added in solution
7	Chelator (C)	(see text)		Added in solution
8	Manure (M)	2%	Commercial steer manure	Added dry
9	N+P+K	[a]		
10	M+P	[a]		
11	M+P+N+K+C	[a]		
12	Leached	(see text)		

[a] Amendments at above level of treatment with phosphorus at 50 ppm.

[b] i) 5 replicates/parameter
ii) spoil material only
iii) all samples limed, 0.7%

Bioassay 3 manure was added because of its better nutritional quality (nitrogen (total N = 2.1 percent), phosphorus (0.37 percent total P, 0.08 percent bicarbonate extractable PO_4-P), carbon input), organic chelation characteristics, as a mulch (soil conditioner), and as a microbial inoculant.

LEACHING - Leaching was also not tried in the first bioassays but was used here to assess the presence of an inhibitory soluble factor. A large sample of raw spoil was placed in a column and leached with an equivalent volume of distilled, deionized water as prescribed by *Jurinak* (Utah State University, personal communication, 1976). Then the leached spoil was air dried and limed before inoculating with algae.

CHELATORS - Although chelators were added, their concentration was poorly controlled and no effects were observed.

Bioassay 3 was run for about six weeks (40 days) under the environmental conditions previously mentioned. The amendments were added just previous to the algal inoculation and subsequently all samples were adjusted to a 20 percent moisture content. This formally marked the beginning of the bioassay (18 December 1975) and accordingly the first color pictures were taken on this date. After three days, the dehydrogenase activity of one replicate of each treatment was measured to have an initial reference point for this particular parameter. This procedure was necessary due to the active microbial respiration of the manure initially. At three weeks (7 January 1976), nitrogen fixation, dehydrogenase activity, and chlorophyll *a* content of one replicate of each treatment was measured. This measurement was used to determine the length of time necessary for significant bioassay results.

The bioassay was terminated after 19 more days (27 January 1976), and the six week analysis was begun. On this date the nitrogen fixation was measured and then the chlorophyll *a* extracted on each of the remaining three replicates. During the first two bioassays, samples were dried under the lights which convert a substantial amount of chlorophyll *a* to pheophytin *a*. Since pheophytin *a* content was subtracted from the analysis, the chlorophyll *a* content which was reported may have been low. For this bioassay fresh, moist samples were analyzed and corrected for pheophytin and, therefore, only relative differences between treatments are directly comparable between the bioassays. Dehydrogenase activity was measured immediately after the samples were air dried.

In all of the bioassays, significant statistical differences in response between treatments were established at $P \geq 0.95$ using a t-test (*Ostle*, 1963). (Appendix A lists values of t for these comparisons in Bioassay 3.) Throughout the discussion of the comparison between growth responses of the different treatments, the word significant refers to $P \geq 0.95$. Least squares analysis for linear and power curves were done using accepted statistical techniques (*Ostle*, 1963).

RESULTS

The bioassay results on the amended spoil samples are presented in this section, first with an overview and then specifically for each parameter; then the parameters are related as an attempt to provide specific functional relationships. Although results are presented on a per gram basis, an areal basis can be calculated by multiplying results by 0.64 to obtain units per $cm^2 \cdot cm$ deep.

For most parameters growth responses determined at three or six weeks can be compared without undue concern for initial variability (Table 3). However, the dehydrogenase measurements show that high microbial activity is associated with the manure.

Overview of Results

The results of the three week and six week measurements of the major microbial response parameters show substantial variation with time and more importantly due to amendments (Figure 2). Growth responses for each treatment correlate very well between the response parameters. For treatments having a stimulatory effect on the microorganisms, the response at six weeks was greater than at three weeks. Accordingly, a lack of response observed at three weeks was also observed at six weeks. In general, phosphorus and/or manure amendments caused the greatest increase in microbial activity in the spoil while nitrogen prevented growth.

Microscopic examination of the various algal growths on each treatment indicated that the addition of nitrogen markedly affected

Table 3. Initial conditions for Bioassay.

Material	pH	Total N mg/g	Total P µg/g	HCO_3^- Extractable P µg/g	Chlorophyll *a* µg/g	Dehydrogenase µf Formazan/g
Raw spoil	3.4	0.05	1130	23	0.001	0.00
Spoil, limed and inoculated	8.0	≈0.05	1130	39	0.03	<1.00[a]
Spoil, limed, inoculated, and manured (2%)	8.0	0.47	1203	55	0.03	≈12.00[b]

[a] Value based on average dehydrogenase activity at 3 days for all treatments without manure.

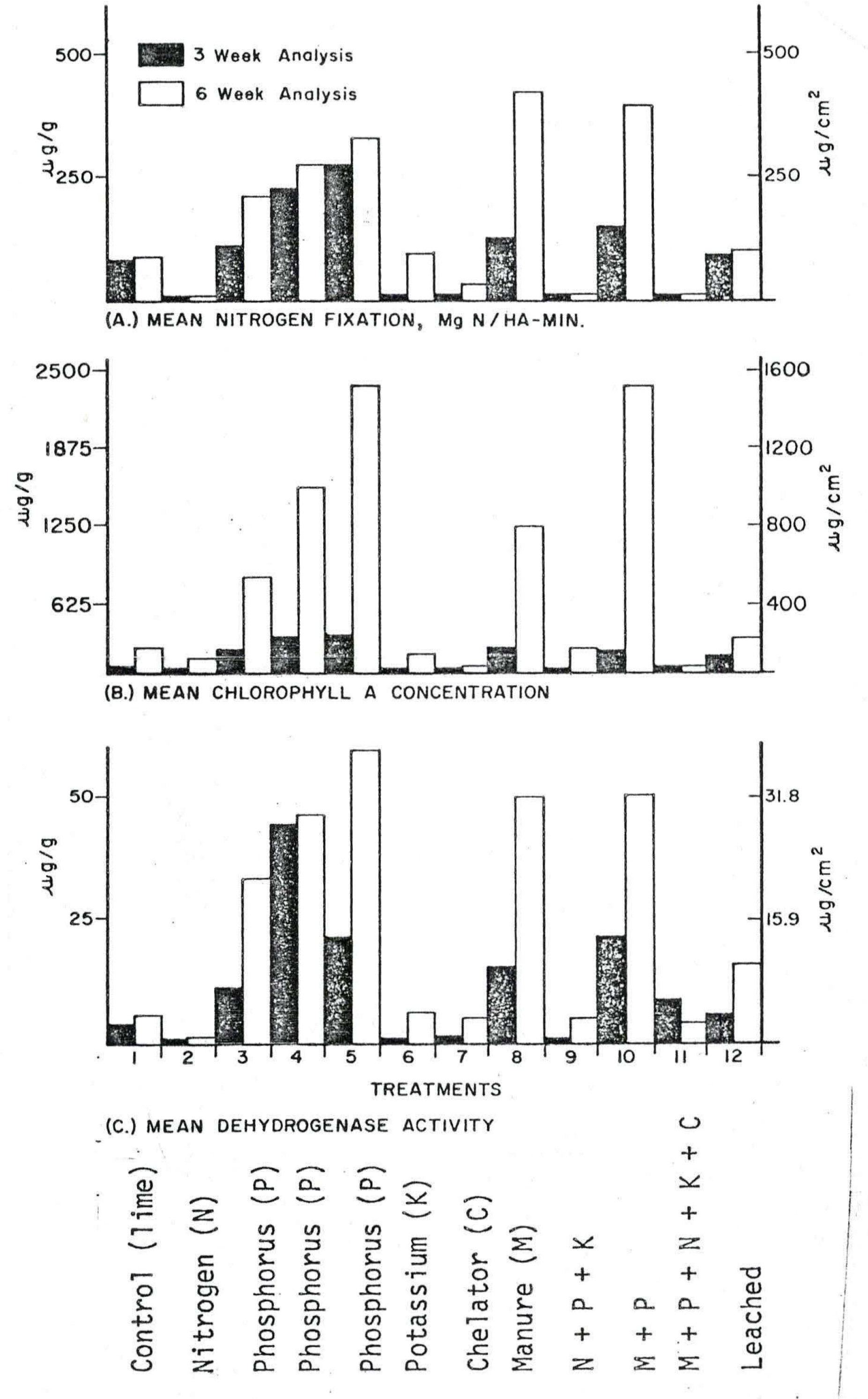
[b] Value based on average dehydrogenase activity of treatments with manure except for treatment 11 which showed no activity. Individual values below.

(i) Initial conditions for all treatments can be inferred from this data and modified for total N and total P based on the known quantities added.

(ii) Nitrogen fixation assumed negligible, initially; measurements in field samples indicate this to be the case.

(iii) Three Day Dehydrogenase Values in Bioassays

Treatment	µg Formazan/g
1	1.1
2	0.6
3	0.2
4	1.9
5	0.9
6	2.8
7	0.1
8	12.2
9	0.7
10	11.7
11	0.1
12	0.5



(A.) MEAN NITROGEN FIXATION, Mg N/HA-MIN.

(B.) MEAN CHLOROPHYLL A CONCENTRATION

(C.) MEAN DEHYDROGENASE ACTIVITY

Figure 2. Growth responses at three weeks and six weeks for each treatment. NOTE: three week results are one analysis, six week results are three replicate analyses.

the algal population present. Generally, all samples where nitrogen was added gave rise predominantly to the growth of *Chlamydomonas sp.* (a green alga). Blue-green heterocytous algae, *Nostoc sp.* and *Anabaena sp.* predominated in the algal community on all samples where nitrogen was not added.

Microbial Responses

Chlorophyll *a*. Chlorophyll *a* on the spoil samples appeared to to be higher in bioassay samples where bicarbonate extractable phosphorus was higher also (Figure 3). The addition of phosphorus and/or manure (for the manure the initial total phosphorus was 0.37 percent and the bicarbonate extractable phosphorus was 770 μg P/g) resulted in chlorophyll *a* production significantly greater than the control (treatment 1). The addition of 100 ppm P (treatment 5) was significantly better than the 50 ppm P (treatment 4) which was significantly better than the 25 ppm P (treatment 3). The addition of manure + 50 ppm P (treatment 10) did not result in significantly better chlorophyll *a* production over the 50 ppm P, 100 ppm P, or the manure addition alone (treatment 8). Treatment 10 was significantly better than the control and the 25 ppm P addition suggesting that phosphorus was longer limiting in the spoil.

Differences in algal growth resulted in high variation in chlorophyll *a* content of manure amended samples; thus, those samples could not be shown to be significantly different from any other treatment. Although the mean value for chlorophyll *a* was higher in the leached treatment (treatment 12) than the control, they were not significantly different. The addition of N, K, and NPK (treatments 2, 6, and 9)

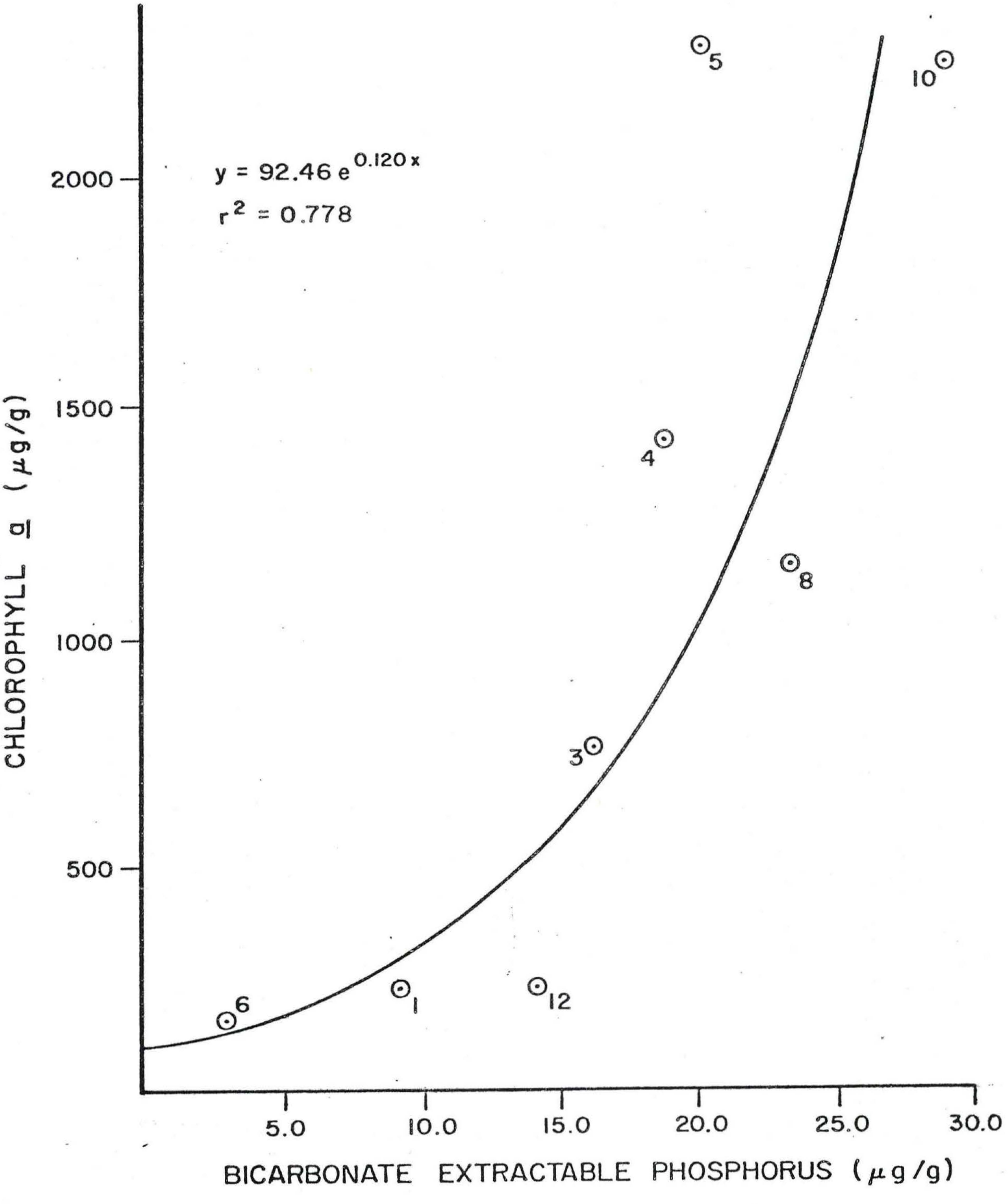


Figure 3. Chlorophyll *a* as a function of bicarbonate extractable phosphorus.

did not produce significantly different chlorophyll *a* than the control. The addition of NPK chelates + manure (treatment 11) and the addition of chelator (treatment 7) were significantly worse than the control. Generally, the addition of N or chelator was seemingly inhibitory to algal growth and the addition of K as K_2SO_4 did not significantly stimulate algal growth over the control.

The three week data for chlorophyll *a* indicated similar relationships as those just defined at six weeks. There was, however, no appreciable chlorophyll *a* production with added nitrogen (treatments 2 and 9) and only half the chlorophyll *a* content of the control was observed with potassium addition, the chelate addition, and the mixed treatment (treatments 6, 7, and 11). The leached sample had more than twice the chlorophyll *a* of the control at three weeks.

Dehydrogenase measurements. The addition of phosphorus and/or manure (treatments 3, 4, 5, 8, and 10) resulted in significantly higher microbial respiration over the control (Figure 2). There were few significant differences between the phosphorus and manure additions with only the 100 ppm P addition (treatment 5) and the manure + 50 ppm P addition (treatment 10) being significantly better than the 25 ppm P addition (treatment 3).

Nitrogen addition (treatment 2) resulted in significantly less growth than any other treatment. The conclusion that the addition of high levels of nitrogen inhibits growth seems probable. Nitrogen addition with other nutrients were the same as the control (treatment 9) or significantly less (treatment 11). Potassium and chelator amendments (treatments 6 and 7) were the same as the control.

Dehydrogenase activity correlated with bicarbonate extractable P measurements (Figure 4) in a similar fashion as chlorophyll *a* (Figure 3).

The leached treatment showed significantly more dehydrogenase activity than the control and this coupled with the mean chlorophyll *a* content being higher than the control (not significantly higher) suggest a possible soluble substance (salt) inhibition.

Manure acted as an excellent microbial inoculant and thus dehydrogenase activity was measured after the first three days so that the microbial responses of the manure addition could be put into perspective. All treatments other than manure (treatment 8) and manure + 50 ppm P (treatment 10) showed minimal microbial respiration at three days. The dehydrogenase activity of these two treatments were 12.2 and 11.7 μg formazan/g, respectively (Table 3). Although the manure treated spoils continued to increase in activity with time during the first three weeks, the dehydrogenase activity that developed from an inorganic phosphorus addition was essentially equal to the samples with manure additions.

The mixed amendment treatment (11) (N, P, K, chelator and manure) showed absolutely no microbial activity initially, was still very poor after three weeks, and was significantly worse than the control at six weeks. It would appear that high levels of nitrogen and/or the combination have a strong inhibitory effect upon microbial activity. The nitrogen apparently reduced the microbial activity added with manure even at three days.

Nitrogen fixation (acetylene reduction). Nitrogen fixation was notably high with the addition of phosphorus and/or manure and expectantly absent with all nitrogen additions. Consistent with those

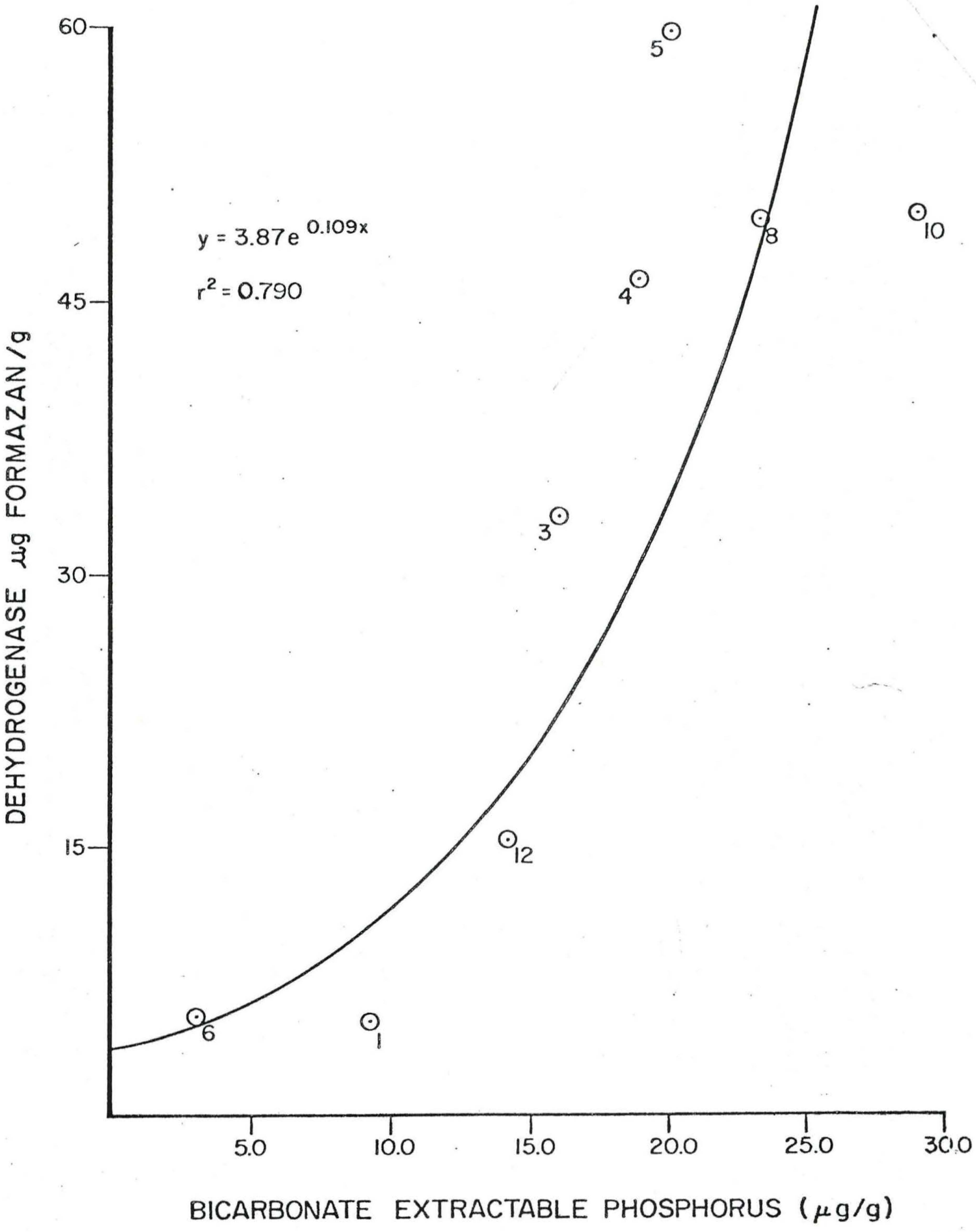


Figure 4. Dehydrogenase activity as a function of bicarbonate extractable phosphorus with best fit power curve.

observations. The algae growing on spoil without nitrogen amendments were 70 to 90 percent heterocystous *Nostoc sp.* The algal material on spoil with nitrogen amendments was about 70 to 100 percent *Chlamydomonas sp.* The remainder for both sets was made up of a diversified group of the heterocystous and non-heterocystous blue-green algae.

All phosphorus additions and/or manure additions produced nitrogen fixation rates significantly better than the control with the rate being related to the bicarbonate extracted phosphorus content (Figure 5). Both the 50 ppm P addition and 100 ppm P addition showed significantly higher rates than the 25 ppm P addition but were not significantly different from each other. The addition of manure alone (treatment 8) was significantly better than all the inorganic phosphorus additions but, perhaps due to experimental variance, the manure + 50 ppm P (treatment 10) was not significantly different from any phosphorus (treatments 3, 4, 5) or manure additions (treatment 8). The control was not significantly different from the potassium addition or chelator addition.

At six weeks total nitrogen was related to nitrogen fixation rates. Both variables increased with higher phosphorus additions (treatments 3, 4, 5, 8, 10) as compared to the control. Thus phosphorus additions, whether with manure or inorganic chemicals, increased both nitrogen fixation rates and total nitrogen concentration. From the third to sixth week nitrogen fixation rates continued to increase on all samples where the nitrogenase activity was measurable, but not to the same extent as the increase from the beginning of the bioassay to the third week.

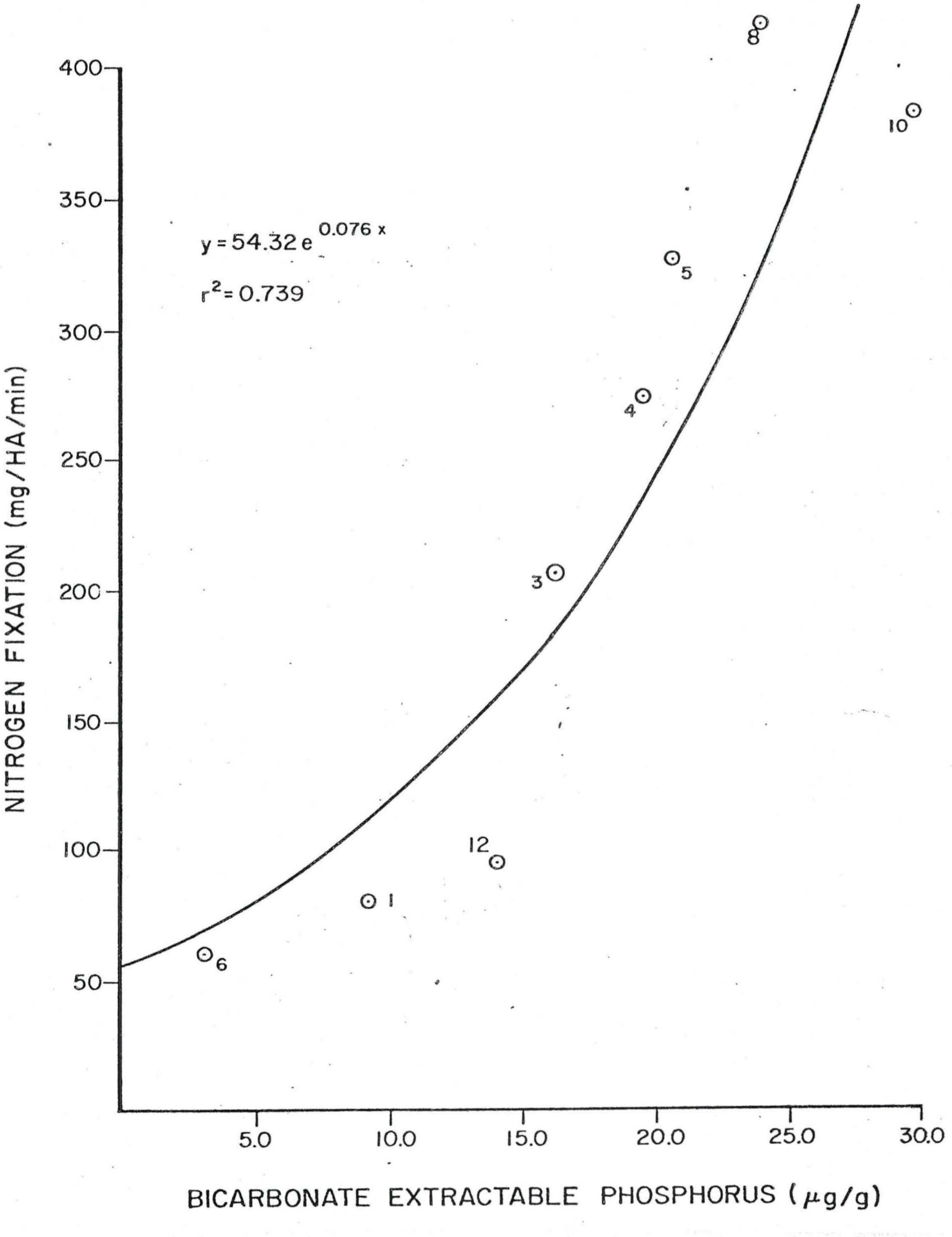


Figure 5. Nitrogen fixation as a function of the bicarbonate extractable phosphorus content with best fit power curve.

Pictorial record. Inspection of weekly slides of the spoil surface in each of the various treatments during this bioassay indicated that phosphorus addition resulted in a more rapid growth (35 mm color slides available at Utah Water Research Laboratory; WG-157-Sorensen). Increasing the amount of phosphorus added to the spoil by inorganic salt or organic (manure) addition, hastened the development of algal growth visible to the eye. The more phosphorus added, the greater the initial growth rate, provided nitrogen was not added also. Accordingly, the first algal colonies appeared on the 100 ppm P addition.

The green algal growth developing on the treatments with nitrogen grew most rapidly during the fifth week. This was reflected in the increased chlorophyll *a* in the nitrogen treatments (Figure 2). The manure and the manure + 50 ppm P also showed a marked increase in algal biomass in the fifth week. This is quite possibly a result of bacterial competition for the phosphorus initially (immobilization) with its subsequent release upon death (mineralization). Overall, the chemical analyses at three and six weeks were very representative of the macroscopic algal biomass observed by photography.

Growth Parameter Interrelationships

The strong similarity in response for the parameters tested suggested the possibility that the parameters are functions of each other. Such is the case when chlorophyll *a*, nitrogen fixation, and dehydrogenase are linearly correlated by a least squares regression analysis (Figures 6, 7, and 8). The relatively high r^2 calculated for each graph leads to the conclusion that the algae are the prime

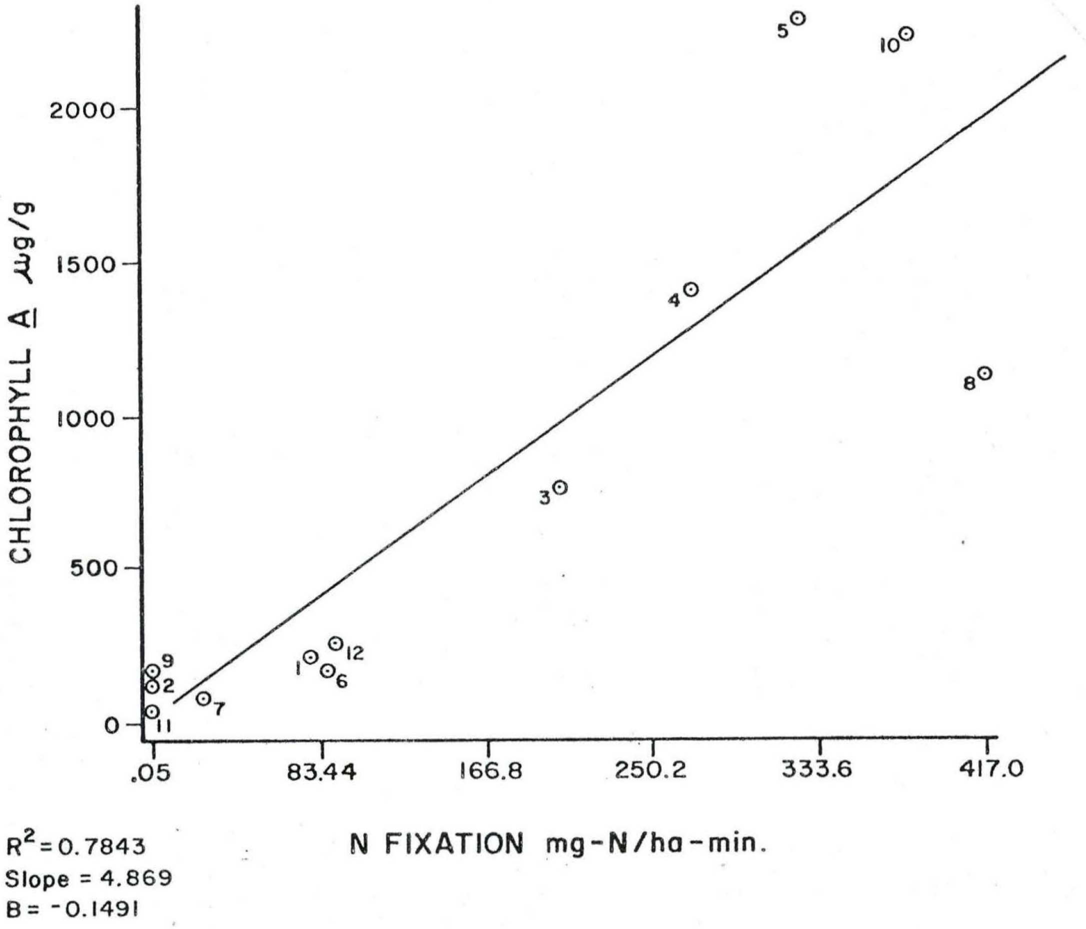


Figure 6. Relationship of chlorophyll *a* to nitrogen fixation.

contributors to the dehydrogenase activity and to the nitrogenase activity (nitrogen fixation). The bacterial contribution to the dehydrogenase activity is either insignificant or effectively masked by the abundant algal population. Secondly, the lower correlation between chlorophyll *a* and nitrogen fixation (Figure 6) is undoubtedly the result of the chlorophyll *a* produced by non-nitrogen fixing green algae. Lastly, the heterocyst, the blue-green algal cell responsible for nitrogen fixation, has an active metabolism substantially greater than vegetative cells (*Carr and Whitton*, 1973). Probably these reasons are responsible for the correlation of dehydrogenase and

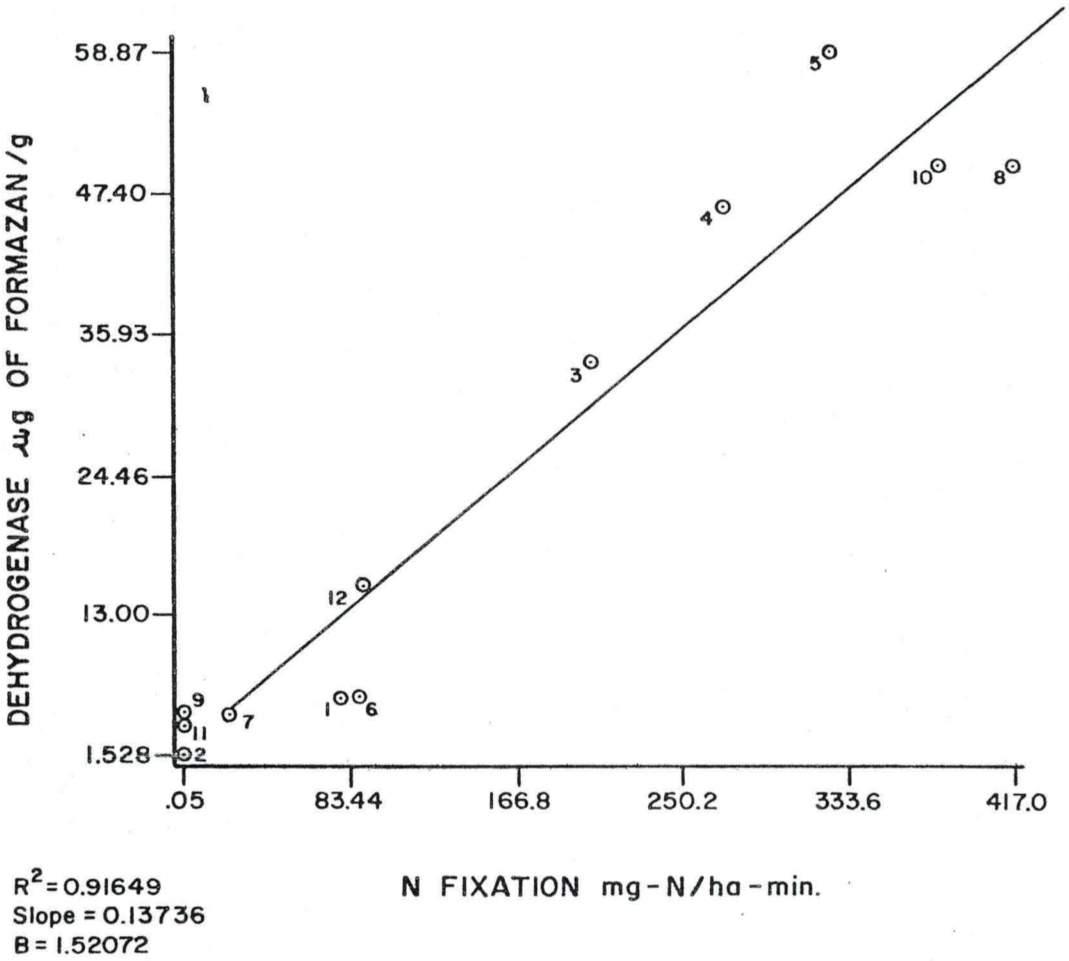


Figure 7. Relationship of dehydrogenase activity to nitrogen fixation.

nitrogen fixation (Figure 7). Again, the slightly lower correlation of chlorophyll *a* and dehydrogenase (Figure 8) is probably due to the contribution of non-heterocystous green algae.

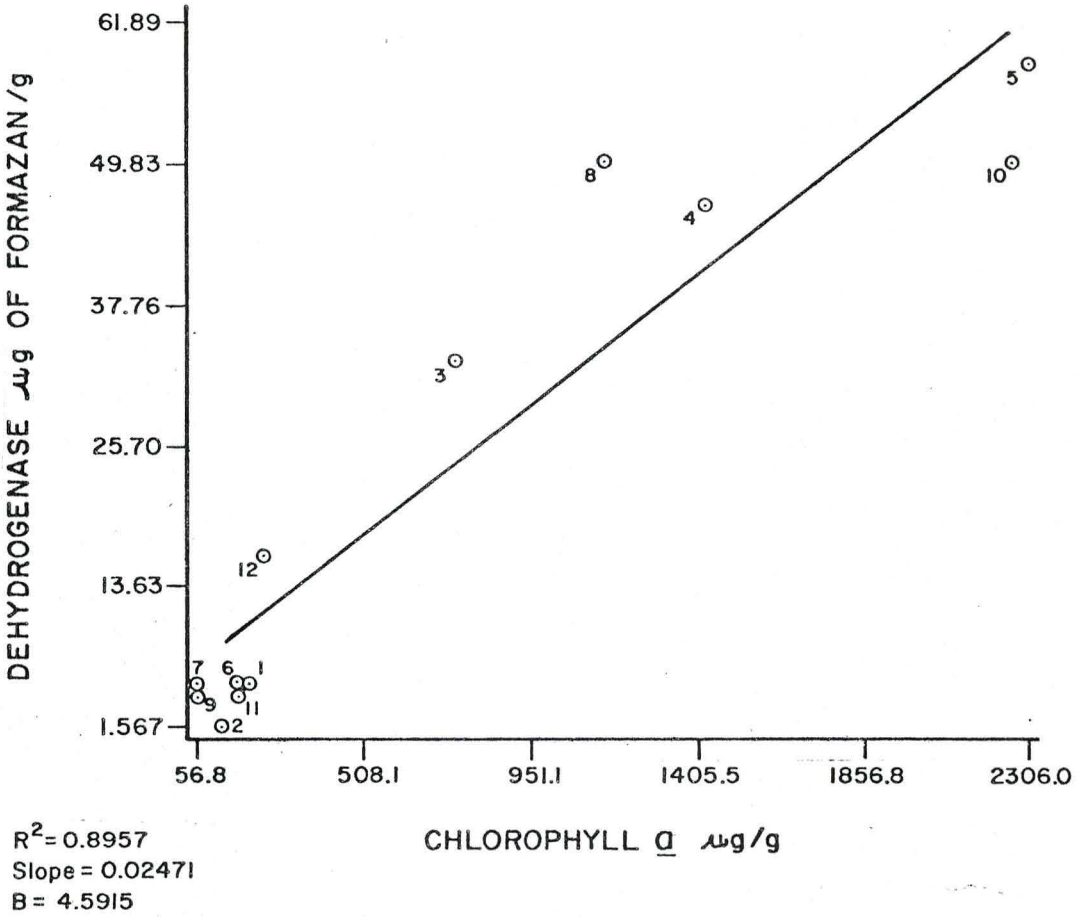


Figure 8. Relationship of dehydrogenase activity to chlorophyll *a* content.

THE FATE OF PHOSPHORUS IN THE SPOIL AND ITS EFFECT ON GROWTH RESPONSE KINETICS

Phosphorus Availability

The form of the phosphate ion has a great effect on the availability of the element to plants. The form is determined by the pH of the soil solution (Figure 9). The $H_2PO_4^-$ form of phosphorus is considered the most readily available to plants but this is further complicated by the presence or absence of other compounds. When phosphates are added to the soil, precipitates of Ca, Fe, and Al phosphates can occur as well as the formation of complexes in the surfaces of calcite, and metal hydroxides (Figure 10).

The initially precipitated compounds revert to more insoluble forms with time, causing the formation of larger crystals and a concurrent decrease in surface area. Furthermore, there is penetration of the adsorbed phosphate onto particles of calcite or metal hydroxides; the phosphate becomes less soluble. Thus, at any time, 80 to 90 percent of the added soluble phosphorus is in a form very slowly available to plants (*Buckman and Brady*, 1969).

The fate of phosphorus in the spoil material is a complex interaction of physico-chemical adsorption on metal hydroxides, calcite, and clay particles, as well as chemical reactions with calcium and iron in solution. The addition of lime alone to spoil results in a substantial increase in the amount of available phosphorus (HC_3^- extractable) (Figure 11). This was inferred to be a result of the solubilization of $FePO_4$ with the rapid increase in pH (*Stumm and*

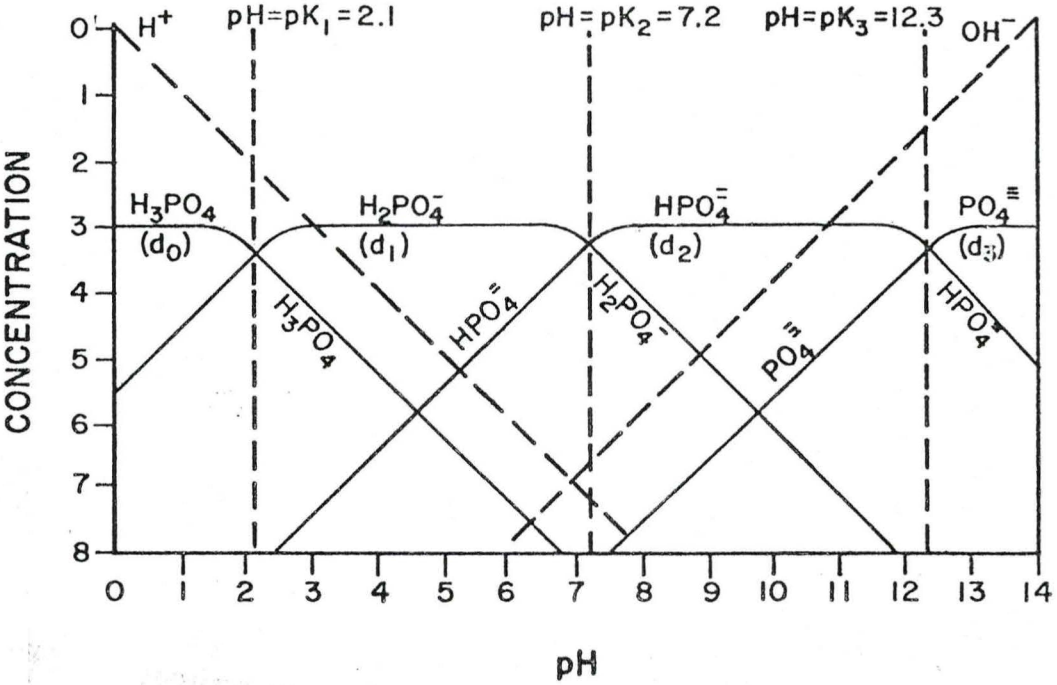


Figure 9. Equilibrium diagram relating pH to the relative concentration of the four phosphate species at C_T = 10^{-3} moles/liter (*Jurinak*, 1976, personal communication, Utah State Univ.).

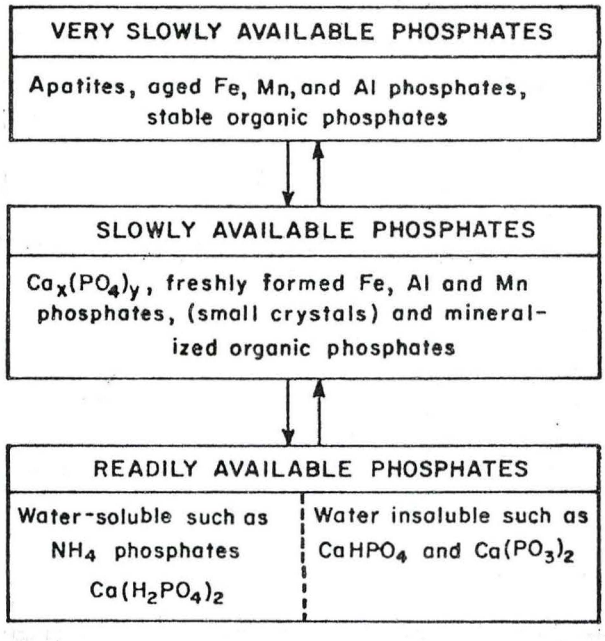

VERY SLOWLY AVAILABLE PHOSPHATES
Apatites, aged Fe, Mn, and Al phosphates, stable organic phosphates
SLOWLY AVAILABLE PHOSPHATES
Cax(PO4)y, freshly formed Fe, Al and Mn phosphates, (small crystals) and mineralized organic phosphates
READILY AVAILABLE PHOSPHATES
Water-soluble such as NH4 phosphates Ca(H2PO4)2
Water insoluble such as CaHPO4 and Ca(PO3)2

Figure 10. Diagram showing the classification of phosphate compounds in three major groups. Fertilizer phosphates are generally in the "readily available phosphate" group but are quickly converted to the slowly available forms. These can be utilized by plants at first but upon aging they are rendered less available and are then classed as very slowly available. At any one time perhaps 80-90 percent of the soil phosphorus is in "very slowly available" form since perhaps less than one percent would be expected to be readily available (*Buckman and Brady*, 1969).

Morgan, 1970). A second inference is the relatively low additional availability of phosphorus for a given incremental addition of phosphorus to the spoil, an observation reflective of the slope of the lines. Third, the sharp increases in extractable phosphorus appear to be points where the addition of phosphorus resulted in a change in the compound controlling its solubility.

The bicarbonate extractable phosphorus data in the bioassay and that in the available phosphorus experiment (Figure 11) were combined to produce the graph shown in Figure 12. The data generated must be viewed in light of the following facts and assumptions:

1. Data at three days were generated from a separate experiment and not the bioassay.
2. The extractable phosphorus at three days for the 100 ppm P addition was extrapolated from Figure 11.
3. The extractable phosphorus of the manure additions at three days is the summation of the available phosphorus of the limed spoil at three days plus the immediately available phosphorus (initial bicarbonate extractable P) of the manure itself.
4. Data at eight days were not a part of the bioassay but was a separate experiment that represents an actual measurement of the extractable phosphorus for the various treatments.
5. Data at 40 days are the mean value of the extractable phosphorus for the various treatments actually measured at the end of the bioassay.

Accepting these assumptions and limitations, it is reasonable that these data approximate the actual changes in extractable phosphorus that occurred during the bioassay. The following mechanisms can be

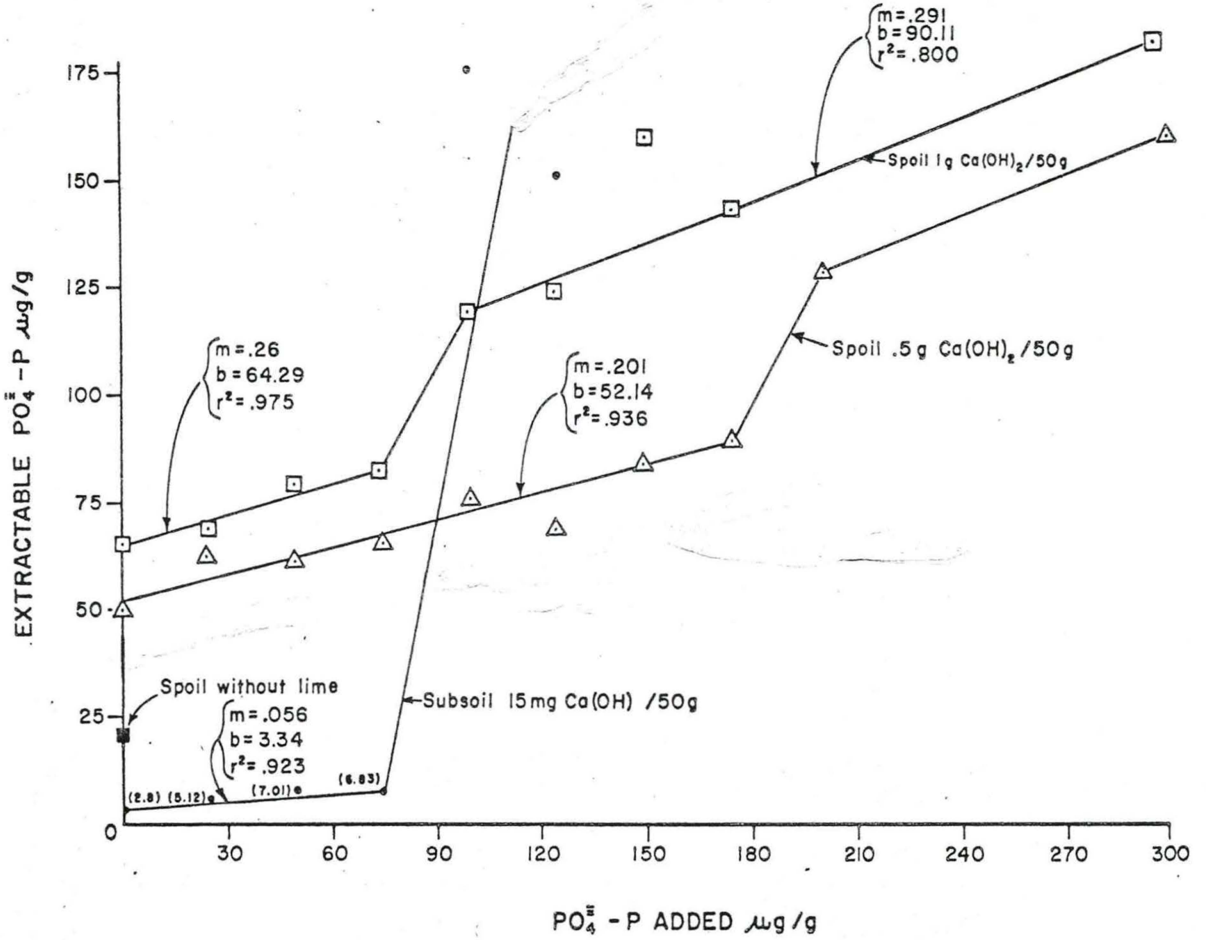


Figure 11. The relationship of HCO_3^- extractable phosphorus to phosphorus addition (as Na_2HPO_4) at various liming levels. Equilibrium time before extraction equaled three days.

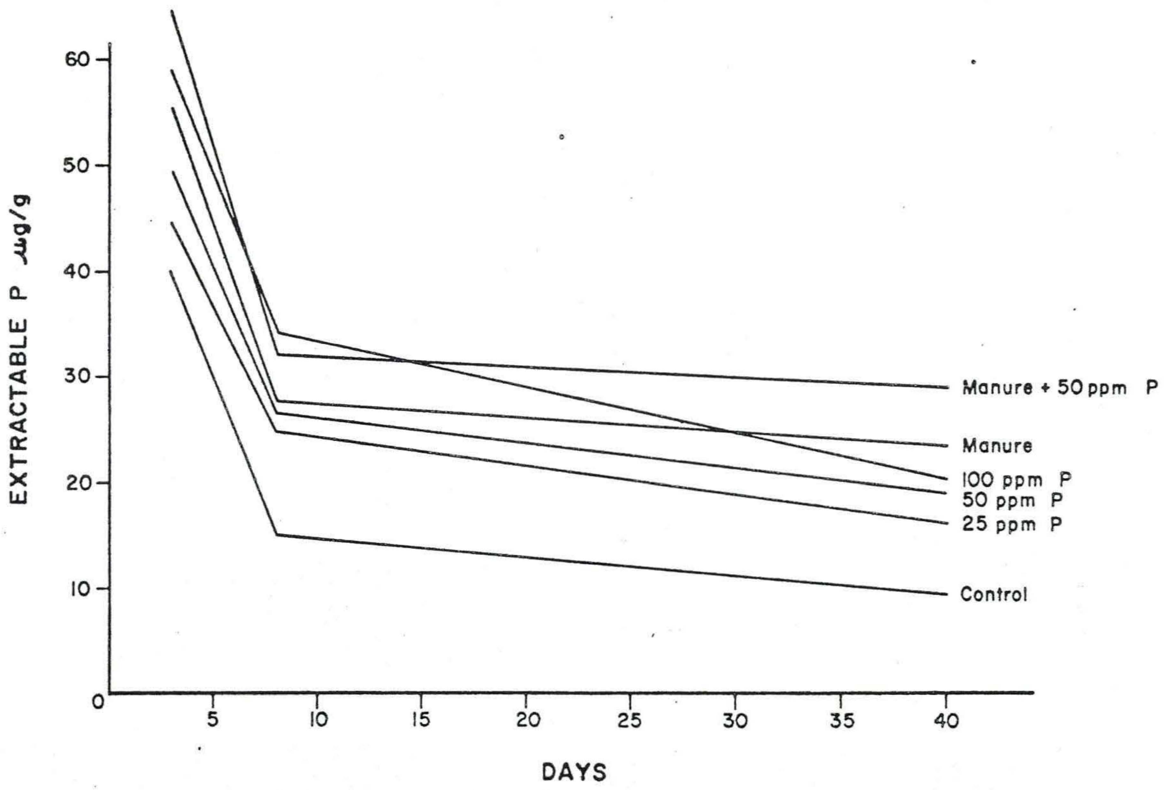


Figure 12. A graphical representation of the availability of phosphorus in the bioassays for various treatments.

inferred based on available literature: In an acid soil (mine spoil), $H_2PO_4^-$ readily reacts with soluble iron and aluminum to form the insoluble compounds of strengite ($Fe(OH)_2H_2PO_4$) and varsicite ($Al(OH)_2H_2PO_4$) respectively. Upon the addition of lime and the resulting rapid rise in pH, these compounds are solubilized releasing a large quantity of phosphorus (Figure 13). This causes the initial rise in extractable phosphorus with the addition of lime after three days. In a matter of five more days the extractable phosphorus has dropped substantially, probably a result of the formation of any number of insoluble calcium phosphate compounds (*i.e.*, dicalcium phosphate dihydrate, octocalcium phosphate, and/or triacalcium phosphate). With further time the extractable phosphorus continues to drop by the formation of very insoluble compounds (*i.e.*, apatite, fluoroapatite, and phosphorus surface complexes of aluminum and iron hydroxides).

To summarize, after lime addition, phosphate is quickly solubilized and then converted to slowly available forms which become less available with time. Furthermore, the graph in Figure 12 indicates the importance of manure in increasing and maintaining the availability of phosphorus. The agricultural practice of fertilizing with phosphorus plus manure (organic matter) resulted in more effective utilization of this element (*Buckman and Brady*, 1969).

Phosphorus Growth Kinetics

The theory or model that has been used the most for understanding microbial growth kinetics is that of *Monod* (1949). A mathematical relationship showing the growth of microorganisms is

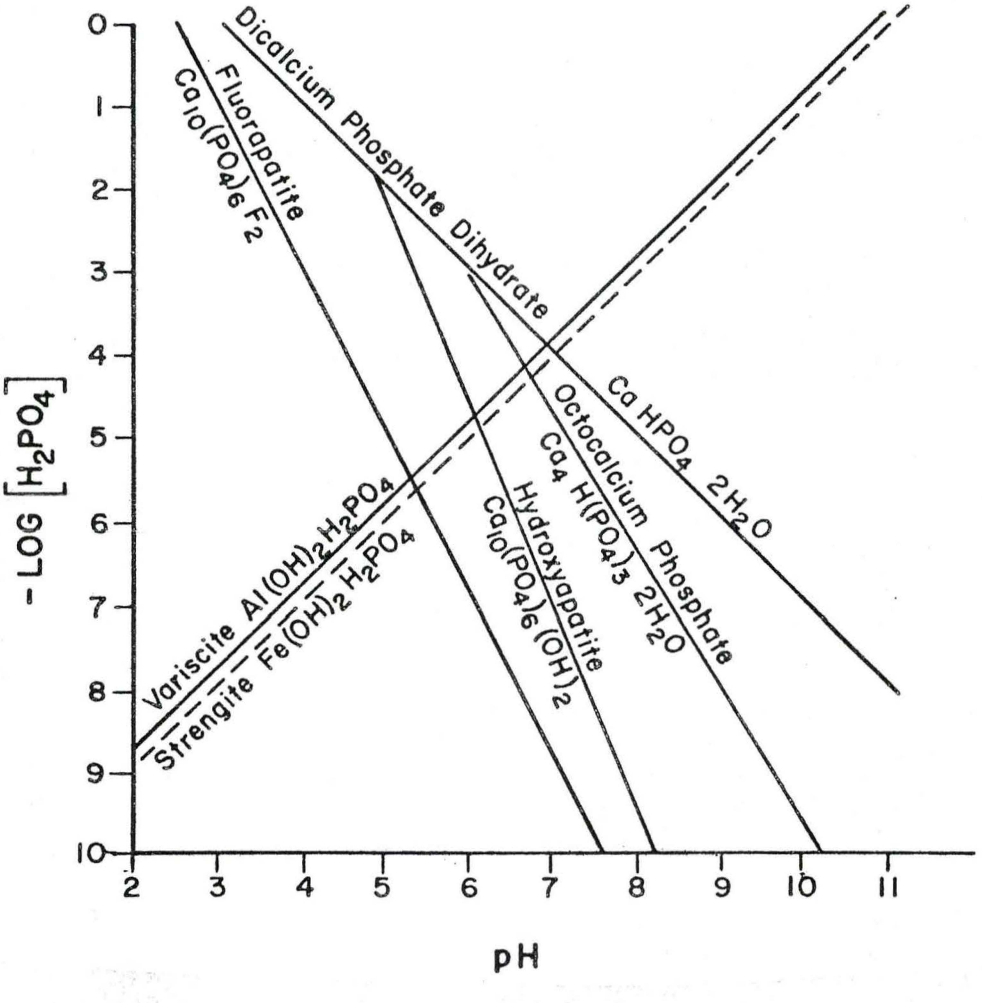


Figure 13. Model showing various compounds controlling the solubility of $H_2PO_4^-$ (moles/liter) as a function of pH (*Jurinak*, 1976, personal communication, Utah State University, Logan).

$$\frac{dX}{dt} = \mu X$$

in which

X = organism concentration (in this case estimated by chlorophyll *a*, dehydrogenase, or nitrogen fixation)

μ = specific growth rate

Upon integration

$$\mu = \frac{\ln \frac{X_2}{X_1}}{t_2 - t_1}$$

The specific growth rate of an organism is dependent on its growth capacity and the environmental conditions present. The specific growth rate is also a function of the limiting nutrient (that element or compound essential for growth but relative to other requirements is in shortest supply) when all other essential elements are in excess

$$\mu = f(S)$$

in which

S = concentration of limiting nutrient (substrate) or factor

A plot of μ versus time for various substrate concentrations yields a series of bell shape curves representing the growth and death of a population as the limiting substrate is assimilated and eventually depleted. The expresstion for the relationship between maximum growth and limiting nutrient concentration is (*Porcella, et al.*, 1970):

$$\mu = \frac{\hat{\mu} S}{K_s + S}$$

in which

$\hat{\mu}$ = the maximum specific growth rate

K_s = substrate concentration producing half the maximum growth rate (half saturation constant)

The assumptions used to apply this model to the Blackbird Mine spoils are:

1. There is one limiting nutrient.
2. That nutrient is phosphorus; more specifically, bicarbonate extractable PO_4-P.
3. The kinetics of interchange between extractable phosphorus and the spoil insoluble phosphorus can be ignored because transfer between these phases is at steady state.
4. Monod-type growth kinetics as described above apply to soil algae.
5. Beacuse of the high water content maintained during the bioassay, the uptake of extractable phosphorus on the spoil sample surface (near the algal growth locus) results in a transport of extractable phosphorus so that effectively the entire mass of spoil extractable phosphorus is available to the surface algae.
6. Treatments of potassium, chelator, leaching and nitrogen are not applicable to this approach due to inhibitory factors not accounted for in the model.

The lack of a well defined system has resulted in these assumptions and some further approximations. First of all, since only three data points for the growth responses were taken with time (initial, three weeks, and six weeks), only two growth rates could be calculated. Initial measurements of chlorophyll *a* after adding the algal inoculum (3 µg/g) and dehydrogenase at 3 days (Table 3) were used; nitrogen fixation rates were estimated from lower limits of detection (0.05 mg N/ha•min). In all cases the calculated growth rate (μ) is higher for the first three weeks (21 days) than for the last three weeks (19 days) (Table 4). It is therefore assumed to be the maximum growth rate. Because uptake of phosphorus by the algae was not measured, yield coefficients with various assumptions about the changes in extractable phosphorus could not be calculated successfully. The extractable phosphorus content at 10.5 days (mid-point of growth curve) was interpolated from Figure 12 for each treatment and was used as the substrate concentration affecting the calculated growth rate. The concentration of phosphorus in the soil water solution is assumed to be a steady state value determined by the relative uptake of phosphorus by the algae and the continued solubilization of phosphatic compounds.

Table 4. Values of μ (days^{-1}) for the bioassay at 10.5 days and at 30 days.

Treatment	Dehydrogenase		Chlorophyll *a*		Nitrogen Fixation	
	@10.5 Days	@30 Days	@10.5 Days	@30 Days	@10.5 Days	@30 Days
1	.059	.020	.147	.058	.350	.003
2[b]	0	.052	.031	.160	0	0
3[a]	.192	.057	.195	.012	.366	.034
4[a]	.185	.002	.217	.090	.400	.010
5[a]	.115	.053	.216	.111	.410	.010
6[b]	-0.159	.447	.098	.102	0	.394
7[b]	.132	.058	.101	.046	0	.333
8[a]	.012	.062	.194	.100	.372	.064
9[b]	-0.093	.199	.016	.198	0	0
10[a]	.027	.046	.194	.133	.380	.050
11[b]	.212	-0.042	.116	.017	0	0
12	.109	.061	.171	.042	.352	.007

[a] Overall response high.

[b] Overall response low or nil.

A plot of μ calculated for N fixation rate and chlorophyll *a* versus extractable P is shown in Figure 14. Dehydrogenase was not plotted due to considerably greater scatter. Values of maximum specific growth rate ($\hat{\mu}$) are 0.4 days^{-1} and 0.2 days^{-1} for nitrogen fixation rate and chlorophyll *a*, respectively. Saturation coefficient (K_s) estimates were 5 μg/g and 10 μg/g of extractable PO_4-P for nitrogen fixation and chlorophyll *a*, respectively.

Even in light of the gross assumptions used to generate this plot, it can at least be concluded that nitrogen fixation responds quickest to the extractable phosphorus content of the spoil as seen by its lower K_s. It is also known that an excess of phosphorus fertilization results in molybdenum increases in plant tissues as much as 10 fold (*Schutte*, 1964) and therefore, molybdenum could limit nitrogen fixation in soil algae.

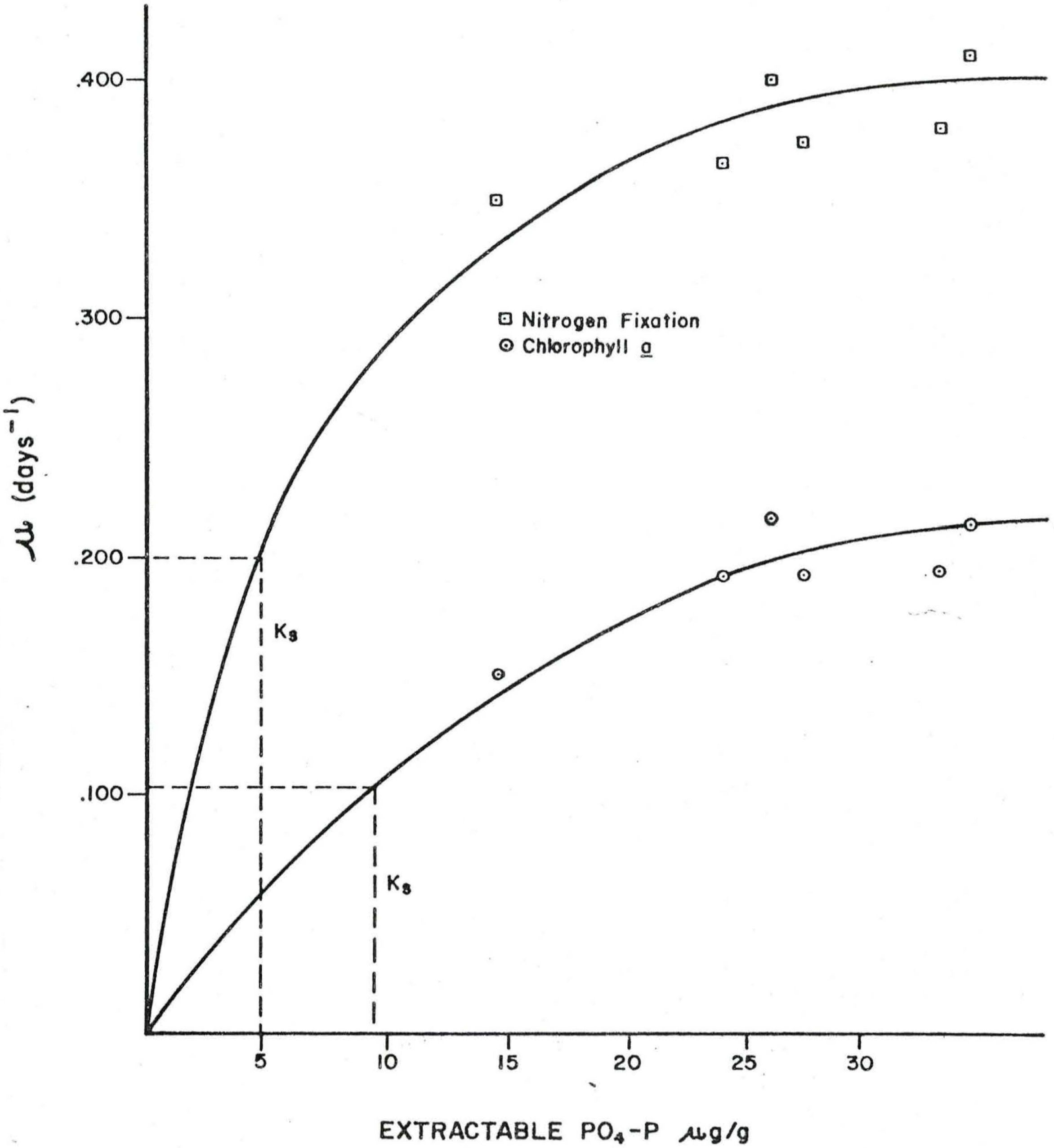


Figure 14. A plot of estimated growth rate versus the extractable phosphorus content at 10.5 days.

DISCUSSION

Microbial Bioassays

The ability of the forest subsoil and raw spoil materials used in the microbial bioassays to support microbial activity is primarily dependent on pH adjustment and control. Water was not allowed to limit growth because it was added in excess of growth requirements.

The first bioassay demonstrated the need for sufficient lime addition to neutralize existing acidity and to consume acid produced by microbial oxidation of sulfide minerals in the material (*Sorensen, et al.*, 1978). The majority of microorganisms that contribute to the fertility of the soil are unable to grow at low pH and are unable to tolerate the high levels of soluble heavy metals in the acid spoil material.

Once the pH had been controlled, the addition of phosphorus to the spoil and subsoil (Bioassay 2) seemed to stimulate microbial development as well as or better than any amendment tried (*Sorensen, et al.*, 1978). The response to manure could not be accurately determined to be a result of any other stimulatory factors aside from the phosphorus input. In all cases, the incremental phosphorus additions (directly or by manure addition) augmented chlorophyll *a* and dehydrogenase activity. The incremental magnitude of the response diminished with increasing phosphorus addition as would be expected when the saturating level of a limiting nutrient is approached. One can conclude that components other than pH control, water, and phosphorus were unnecessary for stimulating microbial growth where nitrogen fixation supplies a satisfactory input to the organic nitrogen pool.

There are distinct advantages and disadvantages of microbial bioassays of soil materials whether natural soils, subsoils, or spoils. Advantages include short time period for study, small space requirements, replication for adequate statistical analysis, analysis of factors related to microbial effects on soil systems (acid production, heavy metal mobilization, anaerobiosis, denitrification, and other processes), application to reestablishment of microbial communities in soil systems and to related questions of soil binding, soil permeability, and erosion relationships. Disadvantages are that algae are not equivalent to plants in terms of nutrient accumulation or composition, nitrogen fixation by plants (legumes and other symbiotic plant nitrogen fixers) probably does not relate closely to quantitative fixation inputs by the blue-green algae, the microbial communities of the shallow soil bioassays are probably more active than in deeper soil profiles.

However, the bioassay shows that microbial establishment takes place quickly under ideal conditions and that nitrogen fixation by plant-bacterial systems is feasible. The establishment of soil microbial populations probably maximizes lasting soil stabilization and revegetation (*Peterson and Nielson*, 1969). At the least the soil bioassay adds a great amount of information on microbial dynamics and allows a significant screening of nutrients which might be valuable in designing a plant-fertilization experiment.

Microbial Role in Fertilization

Nitrogen fixation could add 18.7 lb/acre (21 kg/ha) annually assuming a 60 day growing period with 14 hours of daylight and optimum fixation rates (417 mg/ha•min). This rate occurred with the manure addition (Treatment 8) and was about twice the maximum rate determined from the second bioassay with the addition of 50 ppm P. Ideally, the growth of algae on the spoil in the field as in the laboratory would provide a self perpetuating, low management input of organic matter that would be available for heterotrophic metabolism and humus formation.

The amount of nitrogen added in the laboratory bioassays by the nitrogen fixing algae appeared comparable to inputs in natural terrestial systems (see reviews in *Rychert and Skujins*, 1974; *Sorensen and Porcella*, 1974) and is considerable relative to agricultural applications. Typically, nitrogen additions as fertilizer in cropland in Idaho for a preenergy crisis era are 50-100 lbs/acre and average 26 lbs/acre for all U.S. cropland and total N fertilizer usage (*USDA*, 1967). The Forest Service used amendments of 78 lbs/acre in 1972 and an additional 100 lbs/acre of nitrogen in 1973 in their revegetation experiments and the algal nitrogen fixation inputs would be about 20 percent of each of these additions. Thus microbial reestablishment in disturbed soil systems apparently would have significant value in adding a nutrient which commonly limits revegetation and is increasingly more expensive.

If a microbial population commensurate with the type and magnitude developed in the laboratory could not be established, the seeding of legumes with their associated symbiotic nitrogen fixing bacteria could conceivably add nitrogen in amounts equal to or greater than the annual fertilizer application. Clover was shown to fix approximately 150 lb/acre/yr during a 10 yr experiment on agricultural soil at Ithaca, New York (*Buckman and Brady*, 1969). More pertinently, the low nitrogen content associated with mine spoils is conducive to the use of legumes. Nitrogen accretion by legumes may supply enough nitrogen for grasses without the annual application of a nitrogen fertilizer (*EPA*, 1974). However, legumes are deep-rooted and may finally be inhibited due to heavy metal uptake when their root systems extend below the 2 feet of amended spoil into spoil where toxic levels of metals can have an impact.

The addition of phosphorus in the bioassay was not unlike the rates of phosphorus fertilization at the field level. Typical fertilizer rates for mine spoils are 50-100 lbs/acre (≃25-50 ppm) with Kentucky requiring an absolute minimum of 68 lbs/acre (*EPA*, 1974). In 1972 the USDA Forest Service applied 80 lbs/acre in their revegetation experiments at the Blackbird Mine. The fate of phosphorus added to the soil matrix is a complex interaction of chemical reactions and adsorption phenomena ultimately leading to a reduction in the availability of the added fertilizer. Thus the amount and rate of phosphorus application should be specific for each spoil.

Considering the favorable results of adding 100 ppm phosphorus in this bioassay (≃ 200 lb/acre), the extra cost of this application at the field level may be mitigated through the increased efficacy of the stabilization process.

Although the severe environment at the mine may necessitate more than simple lime, water and phosphorus amendments, blue-green algae should proliferate at the mine site. In arid desert soils, blue-green algae occur as a crust of closely interwoven algal filaments or are found in a lichen association. Their resistance to stressed environments and their lack of competition could allow their growth to become abundant (*Carr and Whitton*, 1973).

SUMMARY AND CONCLUSIONS

The application of soil bioassay technique for determining microbial response (dehydrogenase activity, microscopic observations, nitrogenase activity, chlorophyll *a*, and nutrient availability) to amendments of soil systems (mine spoils, tailings, natural soils, etc.) has been shown to be useful in defining needed soil amendments within a shorter time period than is obtained customarily. The conclusions gained from this study are as follows:

1. Phosphorus was the limiting nutrient for microbial activities in Blackbird Mine spoils. The addition of as much as 100 ppm P continued to show increased growth rate response over smaller additions but was at a saturation level for total biomass production. High phosphorus additions have been shown to significantly promote seedling development (*Peterson and Nielson*, 1969).
2. The addition of manure produced nitrogen fixation rates unexcelled by any other treatment. This has been postulated to be a function of an increased and maintained level of available phosphorus in the spoil.
3. The treatments with the nitrogen amendment (NH_4NO_3) inhibited microbial growth.
4. Chelator and potassium additions resulted in growth responses less than or equal to the control.
5. Leaching of the spoil with a volume-equivalent amount of water produced better growth responses over the control,

indicative of some salinity inhibition.

6. Good linear correlation between various growth parameters is evidence of their value as a technique for microbial analysis of disturbed terrestrial systems.
7. The Walkley-Black technique is invalid for measuring organic carbon in mine spoil samples because sulfide minerals exert a chromate demand.
8. Microbial bioassays can be performed in a short time; responses were reasonably accurate at three weeks relative to six weeks analyses. A four week bioassay would likely be adequate.
9. The development of a microbial bioassay allows understanding of the physical, chemical, and biological interrelationships relevant to the revegetation process on sterile acid mine spoils.

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APPENDIX A

Statistical Comparisons of Response in Bioassay 3

Values of *t* for comparison of response between treatments at 6 weeks (termination) in bioassay 3 are tabulated in this appendix. Values of *t* for comparisons that are not significantly different ($p \geq 0.95$) are not shown. Starred values (***) in the tables represent *t* greater than 9.9. Where the data format is $\overset{a.b}{C}$, a.b is the value of *t* while C is the degrees of freedom in the test. Treatment number identification is shown in Figure 2 in the text.

Table A-1. T-test statistical comparison of total nitrogen content of the various treatments in bioassay 3.

	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	*** 4											
3		7.9 4										
4	3.6 4	7.5 4										
5	3.8 4		3.1 4									
6		*** 4			3.4 4							
7	3.4 4	*** 4			3.3 4							
8	2.8 4											
9	*** 4		8.8 4	8.7 4		*** 4	*** 4					
10	3.2 4		2.8 4			3.0 4	2.9 4					
11	3.6 4		3.3 4	3.0 4		3.4 4	3.3 4					
12	5.5 4	*** 4			2.8 4		3.1 4		*** 4		3.1 4	

Table A-2. T-test statistical comparison of nitrogen fixation rates of the various treatments in bioassay 3.

	1	2	3	4	5	6	7	8	9	10	11	12
1												
2	*** 4											
3	*** 4	*** 4										
4	*** 4	*** 4	4.0 4									
5	*** 4	*** 4	8.0 4									
6		8.5 4	*** 4	9.6 4	*** 4							
7	*** 4	*** 4	*** 4	*** 4	*** 4	5.6 4						
8	*** 4	*** 4	*** 4	7.9 4	5.2 4	*** 4	*** 4					
9	*** 4		*** 4	*** 4	*** 4	8.5 4	*** 4	*** 4				
10	3.2 4	4.0 4				3.1 4	3.7 4		4.0 4			
11	*** 4		*** 4	*** 4	*** 4	8.5 4	*** 4	*** 4		4.0 4		
12		*** 4	*** 4	*** 4	*** 4		*** 4	*** 4	*** 4	3.1 4	*** 4	

Table A-3. T-test statistical comparison of chlorophyll *a* content of the various treatments in bioassay 3.

	1	2	3	4	5	6	7	8	9	10	11	12
1												
2												
3	*** 4	*** 4										
4	*** 4	*** 4	7.0 4									
5	*** 4	*** 4	*** 4	4.7 4								
6			*** 4	*** 4	*** 4							
7	8.1 4		*** 4	*** 4	*** 4	3.2 4						
8												
9			*** 4	*** 4	*** 4		3.0 4					
10	6.5 4	6.7 4	4.7 4			6.6 4	6.9 4		6.5 4			
11	5.2 4		*** 4	*** 4	*** 4	2.9 4			2.9 4	6.9 4		
12			*** 4	*** 4	*** 4		8.3 4			6.3 4	6.0 4	

Table A-4. T-test statistical comparison of dehydrogenase activity of the various treatments in bioassay 3.

```
    1   2   3   4   5   6   7   8   9  10  11  12
 1

 2 5.8
    4

 3 8.5 9.9
    4   4

 4 7.2 7.9
    4   4

 5 3.3 3.6
    4   4

 6     9.6 8.6 7.2 3.3
        4   4   4   4

 7     8.6 8.9 7.4 3.4
        4   4   4   4

 8 6.9 7.5             6.9 7.0
    4   4               4   4

 9     5.7 8.9 7.4 3.4         7.1
        4   4   4   4           4

10 9.2 *** 2.9         9.3 9.5     9.5
    4   4   4           4   4       4

11     4.1 9.1 7.5 3.4 3.1     7.1     9.6
        4   4   4   4   4       4       4

12 *** *** 5.4 5.4     *** *** 5.3 *** 7.1 ***
    4   4   4   4       4   4   4   4   4   4
```